Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells

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The vitamin A metabolite all-*trans* retinoic acid (RA) is an important determinant of intestinal immunity. RA primes dendritic cells (DCs) to express CD103 and produce RA themselves, which induces the gut-homing receptors $\alpha 4\beta 7$ and CCR9 on T cells and amplifies transforming growth factor (TGF)- β -mediated development of Foxp3⁺ regulatory T (Treg) cells. Here we investigated the effect of RA on human DCs and subsequent development of T cells. We report a novel role of RA in immune regulation by showing that RA-conditioned human DCs did not substantially enhance Foxp3 but induced $\alpha 4\beta 7^{+}$ CCR9⁺ T cells expressing high levels of interleukin (IL)-10, which were functional suppressive Treg cells. IL-10 production was dependent on DC-derived RA and was maintained when DCs were stimulated with toll-like receptor ligands. Furthermore, the presence of TGF- β during RA-DC-driven T-cell priming favored the induction of Foxp3⁺ Treg cells over IL-10⁺ Treg cells. Experiments with naive CD4⁺ T cells stimulated by anti-CD3 and anti-CD28 antibodies in the absence of DCs emphasized that RA induces IL-10 in face of inflammatory mediators. The data thus show for the first time that RA induces IL-10-producing Treg cells and postulates a novel mechanism for IL-10 in maintaining tolerance to the intestinal microbiome.

INTRODUCTION

Vitamin A modulates immunity through its active metabolite all-*trans* retinoic acid (RA), which is generated in cells expressing retinal aldehyde dehydrogenases (RALDH) and acts on retinoid receptors in various cell types. Studies utilizing various animal models of vitamin A or retinoid receptor deficiency have revealed an integral role for RA in immunity and tolerance. These studies revealed impaired and/or dysregulated T-cell responses in various models of infection and vaccination strategies.¹ Whereas RA is found at low concentrations throughout the body, RA is present at high concentrations in the small intestine due to metabolizing dietary vitamin A by gut epithelial cells.² In this local environment, RA primes lamina propria (LP) dendritic cells (DCs) to express RALDH2 and become CD103⁺ DCs that produce RA.^{3,4} CD103⁺ DCs are migratory cells that activate naive T cells in mesenteric lymph nodes to become effector T cells that contribute to both intestinal homeostasis and

immunity. A key event in this is that CD103⁺ DC-derived RA ensures the expression of gut-homing integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 by effector T cells.⁵

In addition, the development of Foxp3⁺ regulatory T (Treg) cells has been attributed to the synergistic effects of RA derived from CD103⁺ DCs and bystander transforming growth factor (TGF)- β .^{6–9} This pathway is believed to underlie T-cell tolerance to antigens derived from commensal flora or dietary origin. Another essential immune regulator in mucosal tolerance, however, is interleukin (IL)-10. A key study revealed that IL-10-deficient mice develop lethal colitis in specific-pathogen-free facilities.¹⁰ T-cell activation and colitis are inhibited in IL-10-deficient mice raised under germ-free conditions,^{10,11} demonstrating that IL-10 maintains T-cell tolerance to commensal bacterial antigens. A role of IL-10 in mucosal tolerance is in line with a recent mouse model study¹² indicating that oral tolerance requires T cells expressing $\alpha 4\beta 7$, CCR9, and IL-10. Interestingly, this oral tolerance additionally

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depends on the presence of RA, suggesting a link between RA and the induction of IL-10 in T cells.

Although the relation between RA and IL-10 was investigated in murine models, the link between those two factors in maintaining tolerance in human intestine remains elusive. In mice, Maynard *et al.*¹³ demonstrated that RA actually inhibits IL-10 production, initially induced by TGF- β . Here we show that in a human setting, RA has an opposite role, being important in the induction of human IL-10-producing regulatory $\alpha 4\beta 7^+$ CCR9 $^+$ T cells. Our data show that RA programs RALDH-expressing CD103 $^+$ DCs that generate RA and induce the differentiation of $\alpha 4\beta 7^+$ CCR9 $^+$ T cells that produce high levels of IL-10 and suppress T-cell proliferation. Further analysis shows that the priming of IL-10-producing Treg cells is dependent on RA and is maintained in presence of inflammation. Thus, RA controls T-cell-dependent mucosal tolerance at multiple levels: RA not only induces Foxp3 $^+$ Treg cells in the presence of TGF- β , but also IL-10-producing Treg cells in the absence of TGF- β .

RESULTS

RA-conditioned DCs express CD103, RALDH2, and RA

Mouse model experiments have stressed that LP CD103 $^+$ DCs are the main source of RA to affect the T-cell responses in the mesenteric lymph nodes and to be crucially dependent on RA produced in the gut mucosa. Therefore, a first series of *in vitro* experiments evaluated the effect of RA-priming of human DCs. Human DCs generated from monocytes in the presence of RA indeed did express CD103, the signature molecule of RALDH-expressing murine LP-DCs. These cells did not express CX3CR1 or CCR6, molecules expressed by other local DC subsets (Figure 1a). The induced expression of CD103 is RA specific, as dimethyl sulfoxide used to dissolve RA did not induce CD103 expression (Supplementary Figure 1A online). CD103 expression levels depended on the RA concentration used for DC conditioning (Supplementary Figure 1B). Viability assessment of the RA-conditioned DCs, by propidium iodide staining, revealed slightly decreased cellular viability in comparison with untreated DCs (Supplementary Figure 1C). The RA-treated CD103 $^+$ DCs (RA-DCs) exhibited a moderately elevated expression of the co-stimulatory molecules CD86 and HLA-DR in comparison with conventional monocyte-derived DCs (MoDCs; Supplementary Figure 1D), but RA-DCs fully matured on activation by lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly IC), judged by CD83, CD86, and HLA-DR expression (Figure 1b). This maturation was comparable to maturation of MoDCs, indicating that RA conditioning does not modify DC maturation. Moreover, CD103 expression was not influenced by maturation (Supplementary Figure 2A). In addition, production of IL-12 (Figure 1c) and IL-6 (Supplementary Figure 2B) in response to various toll-like receptor ligands was not significantly different for RA-DCs and MoDCs, but IL-10 production was significantly lower in RA-DCs (Figure 1c).

Murine LP CD103 $^+$ DCs are known for their RA-producing capacity due to their RALDH2 expression. We examined the mRNA expression of *ALDH1A1* and *ALDH1A2*, genes encoding for RALDH1 and RALDH2, respectively, in MoDCs and RA-DCs and found that *ALDH1A2* but not *ALDH1A1* expression is upregulated in RA-DCs (Figure 1d). Compared with MoDCs, RA-DCs also showed a higher activity of total ALDH as determined by Aldefluor assay (Figure 1e and Supplementary Figure 3A). On activation, more RA-DCs demonstrated ALDH enzymatic activity (Supplementary Figure 3A), however, no drastic increase in the total level of activity, reflected by mean fluorescence intensity, was detected (Supplementary Figure 3B). This enzymatic activity was completely abolished using the ALDH-inhibitor diethylaminobenzaldehyde (DEAB; Figure 1e), indicating that the RA-DCs are potent producers of RA. Collectively, these data show that conditioning developing DCs with RA yields a distinct subset that shares key characteristics with murine LP CD103 $^+$ DCs.

RA-DCs induce gut-homing, IL-10-producing Treg cells

To explore the effects of RA-DCs on T-cell responses, we evaluated their ability to induce the gut-homing integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 in an allogenic T-cell model. Indeed, RA-DCs exclusively induced a strong expression of $\alpha 4\beta 7$ in all T cells, independently of the DC activation state (Figure 2a). Moreover, only RA-DCs induced CCR9 in varying percentages of T cells (Figure 2b; isotype controls are shown in Supplementary Figure 4). Interestingly, although RA-DCs expressed equal or even higher levels of co-stimulatory and HLA-DR molecules compared with MoDCs, they were significantly less potent in stimulating naive T-cell proliferation (Figure 2c). The reduced RA-DC-induced T-cell proliferation may be explained by induction of Treg cells. To determine whether DC-primed T cells have regulatory qualities, we tested their capacity to inhibit bystander CD4 $^+$ T-cell proliferation. Indeed, bystander CD4 $^+$ T cells significantly proliferated less in the presence of RA-DC-primed T cells compared with MoDC-primed T cells, which persisted when RA-DCs were activated by LPS or poly IC (Figure 2d).

Surprisingly, compared with those primed by MoDCs, naive T cells primed by RA-DCs did not show significantly enhanced Foxp3 expression (Figure 3a). Strikingly, these T cells produced higher amounts of IL-10, which was 10- or fourfold higher in T cells promoted by LPS- or poly IC-matured RA-DCs, respectively (Figure 3b). To determine whether IL-10-secreting cells were mediating suppression, they were purified using IL-10 secretion assay and fluorescence-activated cell sorting (FACS). The potential of IL-10 $^+$ T cells to suppress the proliferation of bystander T cells was compared with IL-10 $^-$ T cells. The data show that the enhanced suppressor capacity of the RA-DC-primed T cells was entirely confined to the IL-10 $^+$ T-cell population (Figure 3c). This observation indicates a central role for IL-10 $^+$ T cells in the enhanced suppression of RA-DC-primed T cells and further suggests that IL-10 may mediate this bystander T-cell suppression. In contrast to this

Figure 1 RA-conditioned DCs express CD103, RALDH2, and RA. RA-conditioned DCs and MoDC were analyzed before activation by FACS for (a) expression of CCR6, CX3CR1, and CD103 and (b) CD83, CD86, and HLA-DR of RA-DCs and MoDCs after 48 h stimulation with (filled histograms) or without (open histograms) poly IC or LPS. (c) Fold induction of IL-12 and fold reduction of IL-10 (compared with MoDC) after 24 h stimulation with LPS or poly IC of MoDCs or RA-DCs. (d) Fold induction (compared with MoDC) of mRNA expression of *ALDH1A1* ($n=8$) and *ALDH1A2* ($n=11$) in MoDC and RA-DC. (e) Aldehyde dehydrogenase activity of MoDC and RA-DC with or without DEAB as determined by Aldefluor assay ($n=7$). Results are a representative out of four (a), six (b) or the mean $\pm$ s.e.m. of five (c) or seven (e) independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. DCs, dendritic cells; DEAB, diethylaminobenzaldehyde; FACS, fluorescence-activated cell sorting; IL, interleukin; LPS, lipopolysaccharide; MoDC, monocyte-derived DC; poly IC, polyinosinic:polycytidylic acid; RA, retinoic acid.

expectation, neutralization of IL-10 during suppressor assays by antibodies against IL-10 and IL-10 receptors (on target T cells) did not have an influence on suppression (data not shown). In an attempt to determine the suppression mechanism of the IL-10⁺ T cells, other potential mediators of bystander T-cell suppression like TGF- β , CTLA-4, and granzyme B were blocked, but no influence on suppression was observed (data not shown). Thus, although IL-10 identifies a suppressive T-cell population induced by DCs, IL-10 by itself does not mediate suppression in the bystander T-cell suppressor assay.

In spite of their strongly elevated IL-10 production, T cells primed by RA-DCs preserved their ability to produce IFN- γ , as concluded from equal percentages of IFN- γ^+ T cells and the comparable amounts of produced IFN- γ determined by ELISA (**Figure 4a**). To analyze to what extent IL-10 and IFN- γ are

produced by the same cells, we performed an IL-10/IFN- γ co-production assay. Expectedly, RA-DCs induced more IL-10⁺ T cells compared with MoDCs and favor the induction of IL-10 single producers and IL-10/IFN- γ double producers, with a preference for IL-10 single producers rather than IL-10/IFN- γ double producers, although to a lesser extent on poly IC priming (**Figure 4b** and **c**). Taken together, our data indicate that RA conditions DCs to enable T cells to home to the intestinal mucosa and to produce high amounts of immunoregulatory IL-10 without affecting the capacity to produce IFN- γ .

Intestinal homing and IL-10 production depend on RA derived from RA-DCs

To determine to what extent RA-DC-derived RA is responsible for the induction of gut-homing receptors and IL-10, RA signaling during T-cell priming was inhibited by RA receptor blocker

Figure 2 RA-DCs induce gut-homing regulatory T cells. Expression of $\alpha 4$ and $\beta 7$ (a) and CCR9 (b) on T cells primed by MoDC or RA-DC as determined by FACS. One representative FACS plot of six is shown. (c) T-cell stimulatory capacity of MoDCs and RA-DCs as determined by [3 H]-thymidine incorporation, depicted as counts per minute (CPM). (d) Suppressive capacity of T cells primed by MoDC or RA-DC expressed as the percentage of proliferating cells relative to MoDC condition. The lower panel is a representative out of seven experiments. Results are the mean $\pm$ s.e.m. of five (c) or seven (d) independent experiments. * $P < 0.05$, ** $P < 0.01$. CFSE, carboxy fluorescein diacetate succinimidyl ester; DCs, dendritic cells; FACS, fluorescence-activated cell sorting; MoDC, monocyte-derived DC; RA, retinoic acid.

LE540. As expected, RA blockade reduced the expression of $\alpha 4\beta 7$ to levels observed in MoDC-primed T cells and completely abolished the expression of CCR9 (Figure 5a). Surprisingly, RA blockade also significantly inhibited IL-10 production and reduced the percentages of IL-10 $^{+}$ T cells induced by RA-DC-primed T cells (Figure 5b and Supplementary Figure 4), but did not affect IFN- γ production nor the percentages of IFN- γ $^{+}$ cells (Figure 5b and Supplementary Figure 5).

To rule out the possibility that the RA-mediated effects are caused by RA carried over by RA-DCs during DC conditioning and not by genuinely produced RA, ALDH activity was blocked during T-cell priming using DEAB. This ALDH inhibitor was successfully used to block RA-mediated effects caused by RA-conditioned bone marrow-derived murine DCs.¹⁴ Unfortunately, this inhibition did not yield any influence

on the expression of $\alpha 4\beta 7$, CCR9 or IL-10 (data not shown). The lack of influence was caused by the short-lived effect of DEAB. RA-DCs were incubated with DEAB up to 48 h and Aldefluor assay was performed at different time points. As demonstrated in Supplementary Figure 6, the effect of DEAB started wearing off after only 2 h of incubation and completely vanished by 48 h, implying that DEAB is not a proper inhibitor for long-term experiments. We also tried the serum deprivation approach described by Villablanca *et al.*¹⁴ We did indeed see CCR9 expression going down in serum-free conditions, but CCR9 expression was not restored in the presence of retinol (data not shown). CCR9 abrogation in serum-free conditions may be secondary to cellular stress observed in these conditions. Our final approach to answer the issue of RA carryover was by performing T-cell priming by the two different types of DCs in a

Figure 3 RA-DCs induce IL-10-producing regulatory T cells. (a) Percentages of Foxp3⁺ cells were determined by flow cytometry. FACS plots from a representative experiment are shown in the lower panel. (b) IL-10 production by T cells primed by MoDCs or RA-DCs measured on restimulation of resting T cells by anti-CD3/anti-CD28 in 24 h supernatants. The left panel shows the fold induction of IL-10 production compared with the MoDC condition. The right panel is a representative experiment. (c) IL-10 secretion assay was performed on resting RA-DC-primed T cells (day 11) and IL-10⁺ cells were isolated by FACS and used in a suppressor assay. They were compared with IL-10⁻ cells and with the total T-cell population and the T cells primed with MoDC. Results are a representative out of nine (a, lower panel), eleven (b, right panel), and three (left panel) independent experiments or the mean $\pm$ s.e.m. of nine (a, upper panel), eleven (b, left panel), or three (c, right panel) independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001. CFSE, carboxy fluorescein diacetate succinimidyl ester; DCs, dendritic cells; FACS, fluorescence-activated cell sorting; IFN- γ , interferon- γ ; IL, interleukin; MoDC, monocyte-derived DC; RA, retinoic acid.

medium supplemented with ultraviolet (UV)-irradiated fetal calf serum (FCS) to destroy all retinoids present in serum as described by Jaensson-Gyllenback *et al.*¹⁵ Priming naive CD4⁺ T cells by RA-DCs in medium supplemented with UV-irradiated serum did indeed reduce CCR9 expression on primed T cells by $\sim$ 25%, but it did not completely bring it down to CCR9 levels expressed by MoDC-primed T cells (Figure 5c). This strongly implies that the remainder expression of CCR9 is attributed to RA carried over by RA-DCs. Interestingly, adding the RA precursor retinal to T-cell cultures in UV-irradiated serum could indeed rescue the

reduction in CCR9 expression induced by the lack of retinoids in the irradiated serum. Interestingly, IL-10 production by T cells was less dependent on carryover RA. Depleting retinoids in culture medium by UV irradiation of serum reduced IL-10 production by these T cells, which was brought down to the same level as IL-10 production by MoDC-primed T cells. The additional presence of retinal rescues IL-10 production by T cells (Supplementary Figure 7). Thus, although RA-treated DCs have an enhanced expression of *ALDH1A2* mRNA as well as an enhanced RALDH enzymatic activity, RA actively produced by RA-DCs does not completely account for the

Figure 4 RA-DC-induced regulatory T cells maintain their effector cytokine-producing capacity. **(a)** Percentages of IFN- γ -producing T cells was determined by intracellular staining of IFN- γ following restimulation with phorbol 12-myristate 13-acetate/ionomycin in the presence of brefeldin (upper panel). IFN- γ production by T cells primed by MoDC or RA-DC measured on restimulation of resting T cells by anti-CD3/anti-CD28 in 24 h supernatants (lower panel). **(b)** IL-10 co-expression with IFN- γ was determined by IL-10/IFN- γ double secretion assay following overnight restimulation with anti-CD3/anti-CD28. **(c)** The percentages of IL-10 single producers and IL-10/IFN- γ double producers within the whole IL-10 $^{+}$ T-cell population was determined in restimulated cells by the double secretion assay mentioned in **b**. Results are a representative out of four **(b)** independent experiments or the mean $\pm$ s.e.m. of nine **(a, upper panel)**, six **(a, lower panel)** or four **(c)** independent experiments. * $P < 0.05$. DCs, dendritic cells; IFN- γ , interferon- γ ; IL, interleukin; MoDC, monocyte-derived DC; RA, retinoic acid.

Figure 5 Intestinal homing and IL-10 production depend on RA produced by RA-DCs. (a) Expression of $\alpha 4\beta 7$ and CCR9 as determined by FACS in the absence or presence of LE540. (b) ELISA of IL-10 and IFN- γ levels in supernatants of anti-CD3/anti-CD28 restimulated T cells initially primed with RA-DCs and expressed as fold reduction or induction (respectively) in comparison with untreated condition. (c) Expression of CCR9 by MoDC- and RA-DC-primed T cells following culture in IMDM 10% FCS or IMDM 10% UV-irradiated FCS (UV-FCS) alone or with the additional presence of 50 or 10 nM of retinal. (d) Cytokine production of CCR9⁺ expressed as fold induction in comparison with CCR9⁻ T cells (upper panel) with a representative experiment (lower panel). Results are a representative out of six (a, d, lower panel), three (c), or the mean $\pm$ s.e.m. of five (b) or six (d) independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; IFN- γ , interferon- γ ; IL, interleukin; IMDM, Iscove's Modified Dulbecco's Medium; MoDC, monocyte-derived DC; RA, retinoic acid; TGF- β , transforming growth factor; UV, ultraviolet.

observed effects on T cells, which are partially attributed to RA carried over by those DCs.

The full dependency of both CCR9 expression and IL-10 production on RA suggests that both were expressed by the

same T cells. To test this in more detail, T cells primed by RA-DCs were sorted by FACS into CCR9⁺ and CCR9⁻ T cells and analyzed for their ability to produce IL-10 and IFN- γ . Indeed, CCR9⁺ T cells were the main producers of IL-10

(Figure 5d). Also, CCR9⁺ T cells produced more IFN- γ compared with CCR9⁻ T cells, but only twice as much, whereas the difference in IL-10 production is approximately fivefold. This data support the concept that RA provides CCR9⁺ T cells with anti-inflammatory properties.

RA induces gut-homing, IL-10-producing effector CD4⁺ T cells

RA has been shown to enhance TGF- β -induced Foxp3 expression in T cells induced by TGF- β .^{16,17} Indeed, TGF- β addition to co-cultures of naive CD4⁺ T cells and the RA-producing RA-DCs strongly enhanced Foxp3 expression (Figure 6a). Remarkably, adding TGF- β to MoDC/naive CD4⁺ T-cell co-cultures did not have any influence on Foxp3 expression by these T cells. Simultaneously, IL-10 amounts produced by T cells, even those primed by MoDCs, were markedly decreased, suggesting that TGF- β blocks IL-10 production, including RA-induced IL-10 (Figure 6b). TGF- β had no influence on CCR9 induction by RA-DC (data not shown) nor introduced CCR9 expression on MoDC-primed T cells (Supplementary Figure 8). In addition to IL-10, TGF- β inhibited the expression of both IFN- γ by T cells primed by either MoDCs or RA-DCs (Supplementary Figure 5).

To evaluate the direct effect of RA, TGF- β or the combination on the development of IL-10-producing or Foxp3⁺ Treg cells, naive CD4⁺ T cells were stimulated by anti-CD3/anti-CD28 without DCs. These experiments unequivocally confirmed that RA is a unique inducer of IL-10 production in human naive CD4⁺ T cells (Figure 6c). The induction of IL-10 was dependent on the dose of RA. Interestingly, RA-induced IL-10 production persisted during inflammatory conditions, that is, IL-12 presence, which induces the development of Th1 cells from naive precursors. As reported previously, IL-12 by itself induced IL-10, but at modest levels compared with RA. As expected, RA-induced IL-10 was abrogated by the presence of TGF- β (Figure 6d).

Compared with TGF- β , RA is a poor inducer of Foxp3 expression in naive T cells, whereas the combination of RA and TGF- β was superior (Figure 6e). Although IL-12 did not modify RA-induced IL-10 production, it did reduce the number of Foxp3⁺ T cells induced by RA, TGF- β , and the combination (Supplementary Figure 9). These data confirm previously published studies in both mouse models and human *in vitro* experiments showing that RA mainly amplifies TGF- β -induced development of Foxp3⁺ Treg cells. Moreover, IL-12-promoted IFN- γ production by T cells was not modulated by the additional presence of RA (Figure 6f). Altogether, these data

indicate that RA promotes T-cell tolerance through two mechanisms. In the presence of TGF- β , RA amplifies the TGF- β -induced development of Foxp3⁺ Treg cells, which is downregulated in inflammatory conditions. In the absence of TGF- β , RA induces IL-10 production, which is maintained in inflammatory conditions.

Known as a key factor in mediating intestinal homing of T cells, RA did induce the expression of CCR9 on activated naive T cells (Figure 7a). Surprisingly, these CCR9 levels were significantly enhanced on simultaneous RA and IL-12 exposure, although IL-12 alone did not induce CCR9 expression (Figure 7a and b). In contrast, TGF- β did not influence RA-induced CCR9 expression (data not shown). Thus, the ability of RA to prime activated CD4⁺ T cells for the expression of IL-10 and the intestinal homing receptors is preserved, or even amplified, in inflammatory conditions.

DISCUSSION

Intestinal homeostasis is based on a critical balance between tolerance toward innocuous antigens, generously available in the intestine, and immunity toward invasive pathogens. Among the multiple mechanisms present in the intestine to safeguard the host from excessive microbial invasion and inflammation, Treg cells have been implicated as a dominant feature. The main representatives of Treg cells in the gut mucosa are Foxp3⁺ Tregs and IL-10-producing T cells.¹⁸ Foxp3⁺ Tregs are easily induced from naive T cells by the combination of RA and TGF- β .^{6–9} Mouse model experiments have indicated that LP CD103⁺ DCs have a critical role in the decision between regulatory and inflammatory T-cell responses and drive the RA-dependent development of Foxp3⁺ Treg cells in the presence of bystander TGF- β .^{6–9} It is, however, of interest that Foxp3⁻ or TGF- β -deficient mice,^{19–21} as well as patients lacking Foxp3⁺ T cells,²² are not primarily prone to develop severe colitis, suggesting that the role of TGF- β in the maintenance of gut homeostasis may be limited. In sharp contrast, IL-10 is pivotal to maintain immune homeostasis mainly and specifically in gut tissue. IL-10-deficient¹⁰ or IL-10R-deficient²³ mice suffer from severe colitis, following microbial colonization of the gut.^{10,11} Moreover, *IL10* was identified as a susceptibility locus for the development of inflammatory bowel disease in humans,²⁴ and more importantly, patients with a homozygous mutation in either IL-10 or IL-10R suffer from early-onset enterocolitis.²⁵ In addition, CD4⁺ T cell-specific IL-10-deficient mice spontaneously develop colitis, which stresses the crucial role of T-cell-derived

Figure 6 Retinoic acid (RA) induces gut-homing, IL-10-producing effector CD4⁺ T cells. (a) Naive CD4⁺ T cells were stimulated with RA-DC in the presence or absence of TGF- β (10 ng ml⁻¹) and the expression of Foxp3 (a) and IL-10 (b) was determined. (c–f) Naive Th cells were stimulated by anti-CD3/anti-CD28 in the indicated conditions and analyzed for Foxp3 or IL-10 expression. (c) Effect of RA and IL-12 on IL-10 production, expressed as fold induction compared with untreated T cells. (d) The effect of RA and TGF- β on IL-10 production, expressed as fold reduction compared with RA-treated condition. (e) The effect of RA and TGF- β on Foxp3. FACS plots (left) are representative of five independent experiments. A pile up of five experiments (right) shows percentages of Foxp3⁺ T cells with or without one concentration of RA (1 μ M) and TGF- β (1 ng ml⁻¹). (f) Effect of IL-12 and RA on IFN- γ production. Results are a representative out of three (a, b), six (c, right panel), five (e, left panel), or the mean $\pm$ s.e.m. of six (c, left panel), four (d), five (e, right panel), or six (f) independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. DCs, dendritic cells; FACS, fluorescence-activated cell sorting; IFN- γ , interferon- γ ; IL, interleukin; MoDC, monocyte-derived DC; TGF- β , transforming growth factor- β .

Figure 7 Retinoic acid (RA)-induced expression of CCR9 by activated T cells is further boosted in the presence of IL-12. **(a and b)** CCR9 expression by RA-primed T cells in the absence or presence of IL-12. Results are the mean $\pm$ s.e.m. of six **(a)** or a representative out of six **(b)** independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

IL-10 in intestinal homeostasis.²⁶ These data strongly suggest that IL-10-producing T cells may be more important than Foxp3⁺ Treg cells in the maintenance of intestinal tolerance. However, how these cells develop is unclear.

As CD103⁺ DCs are essential in the maintenance of intestinal homeostasis,²⁷ we suspected that they are also important for the induction of IL-10. As we were unable to isolate sufficient numbers of *ex vivo* CD103⁺ DCs, we developed a procedure to generate human CD103⁺ DCs *in vitro*, based on the ability of RA to condition mouse DCs for CD103 expression.²⁸ In this report, we show that the monocyte-derived RA-induced CD103⁺ RA-DCs resemble LP CD103⁺ DCs by harboring high levels of RALDH2 with enzymatic activity. Interestingly, IL-4 and granulocyte-macrophage colony-stimulating factor were also reported to induce RALDH2 expression and function in bone marrow-derived mouse DCs.²⁹ Both cytokines were used in this study to generate human MoDCs, yet high *ALDH1A2* expression and RALDH activity were only observed when RA was added during DC generation. Like mouse and human LP CD103⁺ DCs,³⁰ the *in vitro*-generated RA-DCs induce RA-dependent expression of the gut-homing receptors CCR9 and the integrin $\alpha 4$ and $\beta 7$ subunits on stimulated T cells, linking RA to the development of intestinal immunity. Interestingly, RA-induced CCR9 expression was elevated on additional presence of IL-12, an inflammatory cytokine that by itself primarily induces expression of IFN- γ and not CCR9. The IL-12-mediated amplification of RA-induced gut-homing receptors further stresses the importance of CD103⁺ DCs in promoting intestinal T-cell immunity.

The notion that DC-derived RA promotes either IL-10 secretion or Foxp3 expression by human T cells is surrounded by controversy. Whereas T cells primed by RA-treated DCs were shown to express weakly elevated IL-10 levels, in an RA-dependent mechanism,³¹ another study showed that RA-treated DCs induce Foxp3.³² Here we showed that RA-DCs did

not impressively promote Foxp3 expression in naive T cells, but did induce high levels of IL-10 and immunoregulatory properties in an RA-dependent fashion. However, the presence of bystander TGF- β favored the outgrowth of Foxp3⁺ T cells, corroborating results from earlier studies,^{6–9} and overruled the induction of IL-10⁺ T cells. The paradigm that RA-DC-derived RA induces IL-10 and that bystander TGF- β induces Foxp3 in naive T cells was confirmed in a more simplistic approach by testing the direct effects of RA and TGF- β on naive CD4⁺ T cells in the absence of DCs. The upregulation of IL-10 in RA-primed T cells was not reported in similar studies. However, this lack of enhanced IL-10 is probably due to central variations in the experimental setup³³ or due to the low RA concentration used in such studies (2–10 nM),^{16,17} compared with the range applied in our experiments (10–1,000 nM). Analogous to this, a similar concentration range (100–1,000 nM) of RA was applied to generate bone marrow-derived murine RA-producing DCs, human MoDCs, and for the treatment of murine splenic DCs.¹⁴ Whereas the low RA concentration range meets the physiological RA concentrations in blood,³⁴ the small intestine is the primary site for enzymatic processing of vitamin A into RA and is believed to contain uniquely high concentrations of RA.³⁵ Although it was shown that the RA concentration in murine tissues of the gastrointestinal tract is around 15 pmg^{–1} tissue,¹⁴ it is difficult to translate such measurements to our experimental settings. In tissues, the presence of bioactive substances close to the producing cells, in particular within immune synapses, will be much higher and will account for a need of much higher concentrations of the substance to be present in the culture medium.

Taken together, RA primes RA-DCs for the development of two types of Treg cells. RA promotes the generation of IL-10⁺ Treg cells, whereas RA boosts Foxp3⁺ Treg cells in the presence of bystander TGF- β . This TGF- β could be provided by the epithelial cells present at close proximity of the CD103⁺ DCs.

Alternatively, recent reports showed that mouse CD103⁺ DCs have the unique capacity to activate latent TGF- β into active TGF- β by expressing the β 8 subunit of α v β 8.^{36,37} However, in our experiments, the expression of β 8 on *in vitro*-generated MoDCs and RA-DCs did not significantly differ (data not shown).

An interesting question is to what extent the regulatory properties of CD103⁺ DC are overruled by inflammatory conditions. Activation of RA-DCs by toll-like receptor agonists did enhance IFN- γ , but did not modulate IL-10 expression in the naive T cells. This finding was corroborated in the minimalistic T-cell activation experiments. The concurrent upregulation of IL-10 and IFN- γ is probably meant to allow enhanced IL-10 to keep pace with enhanced IFN- γ to prevent IFN- γ -inflicted excessive damage of host tissue. In contrast, IL-12 inhibited Foxp3 expression induced by RA/TGF- β combination, suggesting that this route of tolerance is inhibited during inflammation. Vice versa, TGF- β is known to suppress the IL-12-driven production of IFN- γ and differentiation of Th1 cells by inhibiting IL-12R β 2 chain expression,³⁸ T-bet,³⁹ and STAT4.⁴⁰ Therefore, limited development of TGF- β -induced Foxp3⁺ Treg cells will not necessarily imply more IFN- γ -induced inflammation. Altogether, the data indicate that RA-DC-derived RA primes naive T cells to produce IL-10 resulting in the development of effector T cells that produce IL-10 with or without IFN- γ . The IL-10-producing T cells are reminiscent of Tr1 cells that can be differentiated *in vitro* with IL-10, and are characterized by a distinct cytokine profile, which includes IL-10, IFN- γ , and IL-5.⁴¹ CD4⁺ T cells coproducing IL-10 and inflammatory cytokines, such as IFN- γ or IL-17, with regulatory properties have also been described^{42–44} and associated with certain pathogens. IL-10 production by effector T cells requires the activation of extracellular signal-regulated kinase (ERK).⁴⁵ Interestingly, RA was reported to activate ERK⁴⁶ insinuating a mechanism by which RA induces IL-10 production in T cells. These reports support the concept that RA-induced IL-10 production in effector T cells reflects a gut-specific negative feedback mechanism that may assure protective T-cell responses.

The mechanism by which RA exerts its effects is not completely clear. Recently, it was demonstrated by Ohoka *et al.*⁴⁷ that *CCR9* gene contains a RA response element (RARE) half site critical for RA-induced promoter activity. Using the MatInspector computer program (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html) we were not able to find a RARE site, or a RARE half site, in close relation to an NFATc2 site in the *IL10* promoter region as is the case for the *CCR9* gene. The fact that the *IL-10* gene does not contain a RARE or RARE half site is also corroborated by the fact that DCs treated with RA do not show enhanced IL-10 production. However, RA has been shown to activate ERK2, one of the important players in relating IL-10 production. Indeed, analyzing the human ERK2 promoter regions, by the aforementioned computer program, revealed a putative RARE-binding site at position 155–179. ERK2 is induced upon T cell receptor ligation, indicating that this may be the mechanism how RA induces enhanced IL-10 production in T cells. Those

potential mechanisms mediating RA effects on T cells may also provide an explanation for the differential dependency of T-cell CCR9 and IL-10 on RA that is carried over by RA-DCs. The dependency on ERK2 for IL-10 expression, which is regulated by both T cell receptor and RA signaling, stipulates a role of RA actively released within the immunological synapse, for example, produced by the DC. On the other hand, CCR9, which is directly regulated by RA, can be readily induced by released RA, including carried over RA, and not necessarily in conjunction with T cell receptor signaling. On the basis of this it may be hypothesized that actively produced RA and carried over RA are located in different compartments within RA-DCs and therefore may have different T-cell imprinting qualities. Further analysis of RA production and localization within RA-DCs is crucial to define the involvement of produced and carried over RA.

Although the experiments using sorted IL-10⁺ and IL-10[−] T cells clearly identified IL-10⁺ fraction of the RA-DC-induced T cells to be the suppressor cells in the classic T-cell bystander suppressor assay, IL-10 did not convey suppression in this assay. This advocates the involvement in this assay of another inhibitory molecule, specifically expressed by this regulatory IL-10⁺ T-cell subset. Unfortunately, neutralization of plausible inhibitory factors, such as TGF- β , CTLA-4, and granzyme B, did not reveal the suppressive mechanism of IL-10⁺ T cells, which is yet to be characterized. The role of IL-10 in mediating direct T-cell suppression is debatable. Whereas IL-10 seems to be instrumental for T-cell suppression exerted by Tr1 cells, it is not important for the suppressive activity of naturally occurring Treg cells.⁴⁸ Indeed, other modes of direct T-cell suppression have been described for Treg cells, including Tr1 cells. Those modes include cell-to-cell contact, which depends on surface inhibitory molecules, and metabolic disruption that is based on generating an immunosuppressive environment.⁴⁹

The observation that the IL-10⁺ Treg cells do not exert their effect via IL-10 in the memory T-cell suppressor assay, therefore, does not imply that this IL-10 has no major role in T-cell tolerance. As mentioned above, IL-10 inhibits the stimulatory capacity, cytokine and chemokine production by antigen-presenting cells.⁴¹ Indeed, supernatants of RA-DC-primed T cells were able to inhibit inflammatory cytokine production by stimulated monocytes (**Supplementary Figure 10**), which was abrogated in the additional presence of neutralizing antibodies against IL-10 and IL-10R, indicating a role for IL-10. This finding is in accordance with the observation that monocytes from early-onset enterocolitis patients with mutations in IL-10R secrete higher levels of inflammatory cytokines in comparison with healthy controls.²⁵ Furthermore, IL-10 can control effector T cells as indicated by a recent report showing that Treg-derived IL-10 controlled the *in vivo* expansion of murine Th17 cells, which expressed high levels of IL-10R.⁵⁰ Thus, IL-10 exerts a multifaceted inhibitory effect on T-cell immunity by both indirect effects via antigen-presenting cells and direct effects on Th17 cell expansion.

Collectively, we identified a novel mechanism by which RA-DC-derived RA contributes to T-cell function. RA primarily

targets naive T cells to the intestine by inducing the expression of gut-homing receptors, which is enhanced by IL-12. This finding unexpectedly indicates that RA is key to maintenance of intestinal homeostasis mediated by IL-10-expressing T cells, which prevents the development of inflammatory bowel disease.

METHODS

In vitro generation and activation of MoDCs and RA-DCs. MoDCs were generated as described elsewhere.⁵¹ In brief, monocytes were isolated from peripheral blood mononuclear cells using density centrifugation, then cultured for 6 days in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Paisley, UK) containing gentamicin (86 µg/ml⁻¹; Duchefa, Haarlem, The Netherlands) and 10% FCS (Gibco), supplemented with granulocyte-macrophage colony-stimulating factor (500 U/ml⁻¹; Schering-Plough, Uden, The Netherlands) and IL-4 (10 IU/ml⁻¹; Miltenyi Biotec, Bergisch Gladbach, Germany). RA-DCs were generated in the additional presence of 1 µM of RA (Sigma-Aldrich, St Louis, MO). Expression of surface molecules was determined using the following antibodies: anti-CD103-biotin (Beckman Coulter, Marseille, France) followed by streptavidin-PE, anti-CCR6-PE (BD Biosciences, San Jose, CA), and anti-CX3CR1-PE (MBL, Nagoya, Japan). Immature DCs were stimulated (48 h) with either 100 ng/ml⁻¹ LPS (*Escherichia coli*) or 20 µg/ml⁻¹ poly IC (both Sigma-Aldrich) and analyzed for the expression of surface molecules using the following antibodies: anti-CD86-allophycocyanin (APC), anti-CD83-phycoerythrin (PE), anti-HLA-DR-peridinin-chlorophyll-protein (PerCP), and anti-CD14-fluorescein isothiocyanate (FITC) (all BD Biosciences).

Aldefluor assay. The ALDH activity of DCs was determined by the ALDEFLUOR staining kit (Aldagen, Durham, NC) following manufacturer's instructions.

Quantitative real-time PCR. DC mRNA was isolated using a RNA isolation kit (Machery-Nagel, Duren, Germany) followed by complementary DNA synthesis (Fermentas, St Leon Rot, Germany). Real-time quantitative PCR was performed on iCycler using SYBR green fluorescence detection (both Bio-Rad, Hercules, CA). Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase. Used primers: *ALDH1A1*, 5'-TGGCTTATCAGCAGGAGTGT-3', 5'-ACCGTACTCTCCAGTTCTCTTC-3'; *ALDH1A2*, 5'-GGTGACCTTTC TCCTGTC-3', 5'-TGCCCCAGAATGAGCTCA-3'; glyceraldehyde 3-phosphate dehydrogenase, 5'-GAAGGTGAAGGTGGGAGTC-3', 5'-GAAGATGGTGATGGGATTTC-3'.

Analysis of DC-derived cytokine production. Immature DCs (20 × 10³) were stimulated with a toll-like receptor ligand: LPS, poly IC, 10 µg/ml⁻¹ peptidoglycan, *Staphylococcus aureus*, Sigma-Aldrich), 2 µg/ml⁻¹ resiquimod (R848), or 1 µg/ml⁻¹ flagellin (*Salmonella typhimurium*; Invivogen, San Diego, CA). The levels of IL-12p70 IL-6 and IL-10 in 24 h culture supernatants were determined by specific solid phase sandwich ELISA.⁵¹

Isolation of naive and memory CD4⁺ T cells. The total CD4⁺ T-cell population was isolated from peripheral blood mononuclear cell by negative magnetic selection using the MACS CD4⁺ T cell isolation kit (Miltenyi Biotec). The naive and memory populations were separated based on CD45RO expression as described previously.⁵²

Stimulation and analysis of naive CD4⁺ T cells. A total of 50 × 10³ CD4⁺ naive CD4⁺ T cells were cultured in IMDM 10% FCS and stimulated with plate-bound anti-CD3 (16A9, 1 µg/ml⁻¹) and soluble anti-CD28 (15E8, 1 µg/ml⁻¹; both from Sanquin Research, Amsterdam, The Netherlands) in the presence or absence of indicated concentrations of RA, TGF-β, and IL-12 (R&D Systems, Minneapolis, MN). Naive CD4⁺ T-cell stimulation by DCs and

subsequent restimulation were done as described previously.⁵² These co-cultures were performed in IMDM 10% FCS and when indicated, 1 µM of the RA receptor blocker LE540 (Wako Pure Chemical Industries, Osaka, Japan) or 10 ng/ml⁻¹ TGF-β were used. Alternatively, naive CD4⁺ T-cell stimulation by DCs was performed in IMDM supplemented by 10% UV-irradiated FCS to destroy all retinoids present in serum.¹⁵ This was performed by exposing the serum to UV light with a measured output of 18 mW cm⁻² for a 25 min, leading to a total exposure of 27 J. When resting (around day 11), T cells were assessed for the expression of cell-surface molecules using the following antibodies: anti-α4-PE (eBioscience, San Diego, CA), anti-β7-FITC (Biolegend, San Diego, CA), anti-CCR9 (R&D systems), followed by goat-anti-mouse-PE (Jackson Immuno-Research, West Grove, PA). Foxp3 expression was determined by intracellular staining using the Foxp3 staining kit (Biolegend) following manufacturer's instructions. In parallel, 100 × 10³ T cells were restimulated with plate-bound anti-CD3 and anti-CD28. Restimulation was done for 16 h followed by an IFN-γ/IL-10 double secretion assay (Miltenyi Biotec) or for 24 h for analysis of IL-10 (BD Biosciences) and IFN-γ (U-Cytech, Utrecht, The Netherlands) in supernatants. Alternatively, resting T cells were restimulated with phorbol 12-myristate 13-acetate/ionomycin in the presence of brefeldin (all Sigma-Aldrich) and the percentages of IL-10- and IFN-γ-producing cells were determined by intracellular staining using anti-IL-10-APC and anti-IFN-γ-FITC (all BD Biosciences). To determine T-cell proliferation induced by DCs, 11 KBq per well [³H]-TdR (Radiochemical Center, Amersham, UK) was added on days 3–5 of co-culture of naive CD4⁺ cells (50 × 10³) with the indicated numbers of DCs. The incorporated [³H]-TdR was measured after 16 h by liquid scintillation spectroscopy.

T-cell suppressor assay. T-cell suppressor assay was performed as described previously.⁵² In short, the T cells induced by co-culture with MoDCs or RA-DCs (test cells) were collected after 5 days, extensively washed, counted, irradiated (30 Gy) to prevent expansion, and stained with the cell-cycle-tracking dye PKH-26 (11.8 µM, Sigma-Aldrich). Memory T cells, from the same donor as test cells, were purified as mentioned above, labeled with 5,6-carboxy fluorescein diacetate succinimidyl ester (0.5 µM; Molecular Probes, Eugene, OR), and subsequently used as bystander target cells. Test cells (50 × 10³) were co-cultured with 25 × 10³ target cells and 1,000 LPS-matured MoDCs in IMDM 10% FCS. After 5–7 days, the proliferation of the target T cells was determined by flow cytometry.

T-cell sorting and flow cytometry. DC-primed T cells were sorted following staining with anti-CCR9 and anti-CD4-APC (BD Biosciences) or after performing IL-10 secretion assay on restimulated T cells. Sorting was performed using FACS Aria (BD Biosciences), and flow cytometry analysis was performed on FACS Canto II (BD Biosciences). Data analysis was done using FlowJo software (Tree Star, Ashland, OR).

Statistics. Student's *t*-tests were performed for paired measurements with GraphPad Prism software (GraphPad, La Jolla, CA). Values of *P* < 0.05 were considered significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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